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The cyanobacterial alkaloid nostocarboline: an inhibitor of acetylcholinesterase and trypsin

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Abstract Preselected cyanobacterial strains (available from culture collections and our own isolates), belonging primarily to the heterocystous cluster, were screened for inhibitors against butyrylcholinesterase. About one-half of the extracts exhibited inhibitory activity. Nostocarboline, the responsible metabolite in *Nostoc* 78–12A, was studied in more detail as an acetylcholinesterase (AChE) inhibitor. The compound showed potent activity against this enzyme (IC_{50} = 5.3 µM), and the Michaelis-Menten kinetics indicated a non-competitive component in the inhibitory mechanism. In addition, nostocarboline turned out to be a potent inhibitor of trypsin (IC_{50} = 2.8 µM), and thus is the first described cyanobacterial serine protease inhibitor of an alkaloid structure. The function of nostocarboline in aquatic ecosystems and its potential as a lead compound for the development of useful therapeutic AChE inhibitors is discussed.

Keywords Screening · Cyanobacteria · Nostoc · Serine protease · Alzheimer's disease

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Introduction

Many secondary metabolites isolated from cyanobacteria show bioactivities and may serve as defensive agents (Burja et al. 2001). By producing such agents, microorganisms minimise the risk of being damaged, out-competed or killed by microorganisms, viruses, inter- and intra-specific competitors and herbivores.

Bioactive cyanobacterial secondary metabolites belong to different chemical classes, such as peptides, lipids, alkaloids and others (Metcalf and Codd 2004; Haider et al. 2003). Among the cyanobacterial alkaloids, some compounds are extremely potent neurotoxins (anatoxins, saxitoxins) exhibiting different modes of action (Carmichael 1992; Metcalf and Codd 2004). One neurophysiological target site is the acetylcholinesterase (AChE) that controls acetylcholine (ACh) concentrations in synaptic clefts by hydrolysing the neurotransmitter ACh into acetate and choline. Potent inhibitory activity was found for anatoxin-a(s), an irreversible inhibitor of AChE, produced by a strain of *Anabaena* (Mahmood and Carmichael 1987). As a consequence of the inhibited hydrolysis of ACh, the excess of neurotransmitter causes maintenance of the depolarisation of the postsynaptic membrane. Symptoms of intoxication in mammals include, amongst others, hypersalivation [suffix (s) in anatoxin-a(s)=salivation], tremors and death. To our knowledge, no other cyanobacterial metabolite has been described as an inhibitor of AChE.

We undertook a screening for cyanobacterial cholinesterase inhibitors with strains that had shown at least slight grazer toxicity (Todorova et al. 1995; Becher and Jüttner 2006). This screening had led to the isolation and structural determination of the alkaloid nostocarboline from the cyanobacterium *Nostoc* 78–12A (Becher et al. 2005). In that previous publication we also described the potent inhibitory activity of nostocarboline on serum butyrylcholinesterase (BChE) and discussed the potential importance of (acetyl)cholinesterase inhibitory β-carbolines for the pathology of Parkinson's disease (PD) and the prospective value of these compounds for the therapy of Alzheimer's disease (AD). We now specify the inhibitory activity of nostocarboline on the key neurophysiological enzyme AChE. As another new feature we also demonstrate the potent serine protease inhibitory activity of this compound. The potential medical applications and the ecological function of nostocarboline as a defensive compound are discussed.

Materials and methods

Cultures (Table 1) were grown under sterile conditions in 300-mL Erlenmeyer flasks as described previously (Becher and Jüttner 2005). Cultures used for experiments were about 6 weeks old when harvested. Cyanobacterial biomass was harvested by centrifugation or by the use of a sieve (polyester, 21 µm mesh width; Sefar, Rüschlikon, Switzerland).

Enzyme inhibition assays

Cholinesterase inhibitory activities were studied for BChE (from horse serum; Sigma, St. Louis, MO) and AChE (from the electric eel, Sigma). Cholinesterase inhibitory and activating effects of cyanobacterial extracts and compounds were determined with a colorimetric procedure based on the Ellman reaction (Ellman et al. 1961). The extraction of cyanobacteria was performed with 60% (v/v) $MeOH/H_2O$. The extracts were dried on a rotary evaporator; the residues obtained were dissolved in Sørensen phosphate buffer (67 mM; pH 7.2). The Sørensen buffer was also used for dissolving the enzymes, substrates and inhibitors. For each assay, an extract equivalent to 65 mg wet cyanobacterial biomass was dissolved in 160 µL Sørensen buffer. When the solution was turbid or contained particles, the suspension was filtered through a 0.45-µm syringe filter (nylon; Semadeni, Ostermundingen, Switzerland) to avoid light scattering in the subsequently performed colorimetric assays.

To test inhibitory activity on BChE, a mixture of the substrate butyrylthiocholine iodide (5 mM), the indicator reagent 5,5'-dithio-bis-2-nitrobenzoate (DTNB, 0.25 mM) and buffer (pH 7.2) [Sigma Diagnostics Cholinesterase (BTC) reagent, no longer available (2006)] was used. For each assay, 60 µL of this reagent, 30 µl BChE (1–15 mU) and 160 µl dissolved extract or inhibitors were added to 350-µl microwells of a transparent 96-well polystyrene plate (Corning Inc., Corning, New York). The synthetic di (hydromethyl)dihydroxypyrrolidine (DMDP) tested for BChE inhibition was obtained from Sigma.

For detailed testing of the AChE inhibitory activity of nostocarboline, acetylthiocholine iodide (ATC, 5 mM; Fluka, Buchs, Switzerland) was used as the substrate. To study the inhibition kinetics, different concentrations of the substrate (S) were applied (0.03–0.6 mM ATC) in the presence of different inhibitor concentrations. DTNB

Table 1 Designation and origin of the investigated cyanobacteria

Cyanobacterial strain assayed	Culture state[a]	Identification number in a culture collection; designation of identical strain[b]	Origin of the strain[b]
Cylindrospermum sp.	ax	ATCC 29412	ATCC
Fischerella sp. (43239)	ax	ATCC 43239	ATCC
Nostoc sp. (78–12A)	ax	ATCC 43238; *Anabaena* sp. (78–12A)	MSU
Nostoc sp. (31)	ax	ATCC 43529	MSU
Fischerella sp. (1829)	ax	UTEX 1829	MSU
Aphanothece sp.	m		Taiwan
JU 5 (LPP group)	m		Türler See
Calothrix anomala	m	SAG 1410–4	SAG
Calothrix thermalis	m	SAG 37.79	SAG
Calothrix sp. (7507)	ax	PCC 7507	PCC
Fischerella cf. *major*	m	EAWAG 108a	Spiez

[a] Axenic (ax) or monoxenic (m)

[b] *ATCC* American Type Culture Collection, Rockville, MD; *UTEX* Culture collection, University of Texas, Austin, TX; *SAG* Sammlung von Algenkulturen, Göttingen, Germany; *PCC* Pasteur Culture Collection, Paris, France; *EAWAG* Eidgenössische Anstalt für Wasserversorgung, Abwasserreinigung und Gewässerschutz, Dübendorf, Switzerland; *MSU* Cultures were obtained from C.P. Wolk, Michigan State University, East Lansing, Michigan USA; *Taiwan* cyanobacterium from Taiwan (Jüttner and Wu 2000) were collected and isolated by F. Jüttner; *Türler See* JU 5 was collected and isolated by F. Jüttner from a biofilm that developed in the littoral zone of Türler See, Switzerland; *Spiez* Culture was obtained from C. Beuret, Spiez Laboratory, Switzerland

(Fluka) was applied in the same concentration as in the BChE assay (0.25 mM). For each assay with AChE, 60 μL ATC, 60 μL DTNB, 30 μL AChE (ca. 1.5 mU) and 100 μL dissolved synthetic nostocarboline (Becher et al. 2005), or buffer in the case of controls, were added to a microwell of a 96-well polystyrene plate.

Colorimetric measurements (at 405 nm) were performed on a SpectraMax 190 multichannel spectrophotometer (SpectraMax, Molecular Devices, Sunnyvale, CA) at 30 °C. Three replicates were measured for each inhibitor of the extract or pure compound and the substrate concentration. The reaction velocity (v) was calculated from the slope of the initial velocity (mean of three replicates). Inhibition and activation were determined in relation to 100% activity controls.

Analyses of the inhibitory activity on trypsin of synthetic nostocarboline were performed with a fluorometric enzyme assay (Baumann et al. 2007). The fluorescent enzyme substrate (Kawabata et al. 1988) was measured on a fluorescence plate reader (SpectraMax Gemini XS, Molecular Devices) in black polystyrene 96-well microtitre plates (Corning). For trypsin inhibition experiments, dimethyled trypsin from porcine pancreas (proteomics grade, Sigma T6567) was used; 5 μL trypsin (67 mU), 145 μL Tris/HCl buffer (50 mM Tris/HCl, 150 mM NaCl, 1 mM $CaCl_2$, 0.1 $mg \cdot mL^{-1}$ bovine serum albumin, pH 8.0) and 30 μl inhibitor solution (in ten different concentrations in the range of 12.5 nM–47 μM final concentration in 200 μL) were preincubated for 5 min at 37 °C. To start the reaction, 20 μL substrate solution [50 μM Boc-Gln-Ala-Arg-7-amido-4-methylcoumarin (Bachem, Bubendorf, Switzerland) in Tris/HCl buffer] were added, and the fluorescence change (λ_{ex}380 nm, λ_{em}440 nm) was monitored for 20 min at 37 °C. Different amounts of nostocarboline were tested in three replicates, and determination of inhibitory activity was similar to that described for cholinesterase.

Toxicity assay

Acute toxicity assays with *Thamnocephalus platyurus* were carried out for 1, 10, 25, 50 and 100 μM nostocarboline solutions as described by Blom et al. (2003). The same molarities were also tested on 10–14 chironomid larvae that were collected from the littoral zone of Lake Zürich, Switzerland.

Results

Screening of cyanobacterial extracts for BChE inhibitory activity

Moderate inhibitory effects were found for extracts of the cyanobacteria JU 5, *Fischerella* (43239) and *Fischerella* cf. *major* (Table 2). Because *Fischerella* (43239) is a producer of hapalindoles exhibiting acute insecticidal toxicity (Becher et al. 2007), we tested the hapalindole-containing C18 HPLC fraction for inhibitory activity on BChE. No inhibition was found for this fraction isolated from an extract of 100 mg wet biomass. The fraction was tested both as an aqueous (the equivalent of 100 mg wet biomass/ 250 μL test volume) and a (4% v/v) methanolic solution (the equivalent of 100 mg wet biomass was dissolved in 10 μL methanol/250 μL test volume). Low inhibitory effects on BChE activity were observed for extracts from *Nostoc* (31) and *Calothrix anomala*. Extracts of *Calothrix thermalis* and *Fischerella* (1829) did not show any inhibitory effects. *Calothrix* (7507) and *Aphanothece* sp. showed increased activities of BChE compared to the 100% controls. *Cylindrospermum* sp. exhibited the highest inhibitory activities against BChE. However, when DMDP (1 mM), an effective digestive glucosidase inhibitor isolated from this strain of *Cylindrospermum* sp. (Jüttner and Wessel 2003), was studied, no inhibitory effect on BChE was found. Another strain with high inhibitory activities was *Nostoc* (78–12A) (Fig. 1). This activity could be attributed to the alkaloid nostocarboline (Becher et al. 2005).

Inhibitory activity of nostocarboline to AChE

The synthesized compound nostocarboline iodide (Fig. 2) was used to determine the inhibition of AChE in more detail. The AChE was from electric eel and its Michaelis constant was calculated to be K_m = 0.07–0.11 mM. This was consistent with the values of 0.11–0.22 mM reported by Mahmood and Carmichael (1987). Nostocarboline exhibited a concentration-dependent inhibitory activity against AChE (Fig. 3a). The concentration of nostocarbo-

Table 2 Modulation of butyrylcholinesterase (BChE) activities by crude extracts of different cyanobacterial strains. The activation and inhibition are calculated from the initial velocities (0–15 min)

Cyanobacterial strain	Activation (%)	Inhibition (%)
Control without extract	0	0
JU 5 (LPP group)	0	68
Cylindrospermum sp.	0	89
Nostoc (78–12A)	0	85
Nostoc (31)	0	18
Fischerella cf. *major*	0	47
Fischerella (43239)	0	41
Fischerella (1829)	0	0
Calothrix anomala	0	24
Calothrix thermalis	0	0
Calothrix (7507)	47	0
Aphanothece sp.	12	0

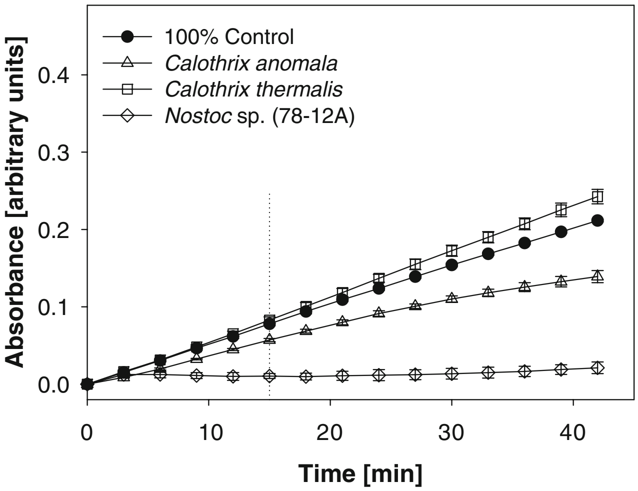

Fig. 1 Modulation of butyrylcholinesterase (BChE) activity (as measure of absorbance) by three selected cyanobacterial crude extracts (mean values and standard deviations calculated from three replicates)

line required to obtain half-maximal inhibition of AChE was IC_{50} = 5.3 μM (SE ± 0.7). To study the inhibition kinetics of nostocarboline for AChE, the initial enzyme rates (v) were assayed with a series of substrate concentrations in the presence of different inhibitor concentrations. The Michaelis-Menten plot (Fig. 3b) showed hyperbolic curves with decreasing maximal velocities (v_{max}) at increasing concentrations of nostocarboline. The double reciprocal Lineweaver-Burk plot (Fig. 3c) showed an interception point of the regression lines left of the [$1/v_{max}$] axis and close to the [1/S] axis, while a slightly negative [S/v_{max}] value of the interception point of the regression lines can be seen in the Hanes plot (Fig. 3d).

Inhibitory activity of nostocarboline on trypsin

When nostocarboline was tested as an inhibitor against porcine pancreas trypsin, strong inhibitory effects were observed. From the regression curve for the concentration dependent inhibition (Fig. 4), an IC_{50} value of 2.8 μM (SE ± 0.2) was calculated.

Toxicity assay

Moderate toxic effects were found for synthetic nostocarboline when it was tested on the crustacean *Thamnocephalus platyurus*. Mortality was 13–20% at 0–10 μM, 27% at 25 μM, 33% at 50 μM and 100% at 100 μM nostocarboline after 24-h testing of 30 animals per molarity. No mortality was found when nostocarboline was tested at a 50 μM concentration on chironomids collected from biofilms of the littoral zone of Lake Zürich, and only two out of ten larvae died at 100 μM concentration.

Discussion

We tested 11 axenic and monoxenic cyanobacterial strains in our culture collection for their inhibitory activity against BChE. Seven of the crude extracts exhibited at least some inhibitory activity. Because bioactive compounds have been described before from some of these strains, the available compounds were also tested for BChE inhibitory activity. Only nostocarboline showed inhibitory activity to BChE (Becher et al. 2005), while the other bioactive compounds were negative. The high inhibitory activity of nostocarboline prompted us to study the inhibitory properties of this compound in more detail.

Although most known cholinesterase inhibitors act against both BChE and AChE, some inhibitors have been found to be selective for only one of these two enzymes (Taylor 1991). When nostocarboline was tested as an inhibitor for AChE (IC_{50} = 5.3 μM) its activity was even stronger than that for serum BChE (IC_{50} = 13.2 μM). The slightly positive $1/v_{max}$ value of the interception point of the regression lines in the Lineweaver-Burk diagram and the slightly negative S/v_{max} value of the interception point of the regression lines in the Hanes plot indicate a non-competitive inhibition with a negative influence on the substrate by nostocarboline (and vice versa) when binding to the enzyme (mixed-inhibition, Bisswanger 2000).

Nostocarboline belongs to the chemical class of the β-carbolines, which are frequently found to be potent bioactive compounds. β-Carbolines affect the endocrine- and nervous system and modulate the functionality of adrenalin, dopamine and serotonin via inhibition of monoamine oxidase. In addition, β-carbolines from plants are believed to cause hallucinations by binding to serotonin receptors (reviewed by Robinson et al. 2003).

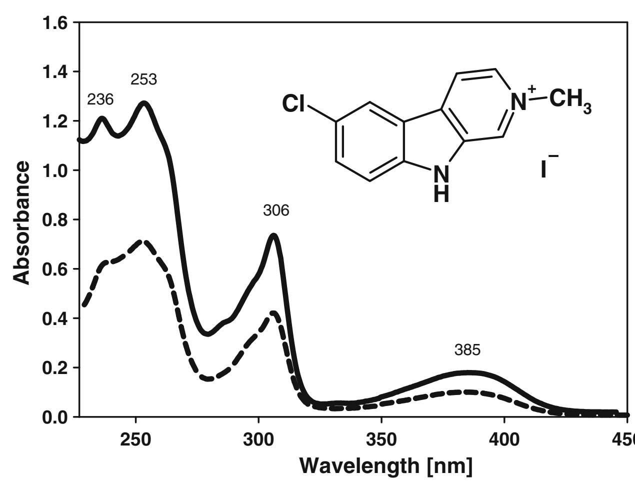

Fig. 2 Structure and absorption spectra of natural nostocarboline [in 60% (v/v) aqueous MeOH; *dashed line*] and synthetic nostocarboline iodide (in 100% MeOH; *solid line*)

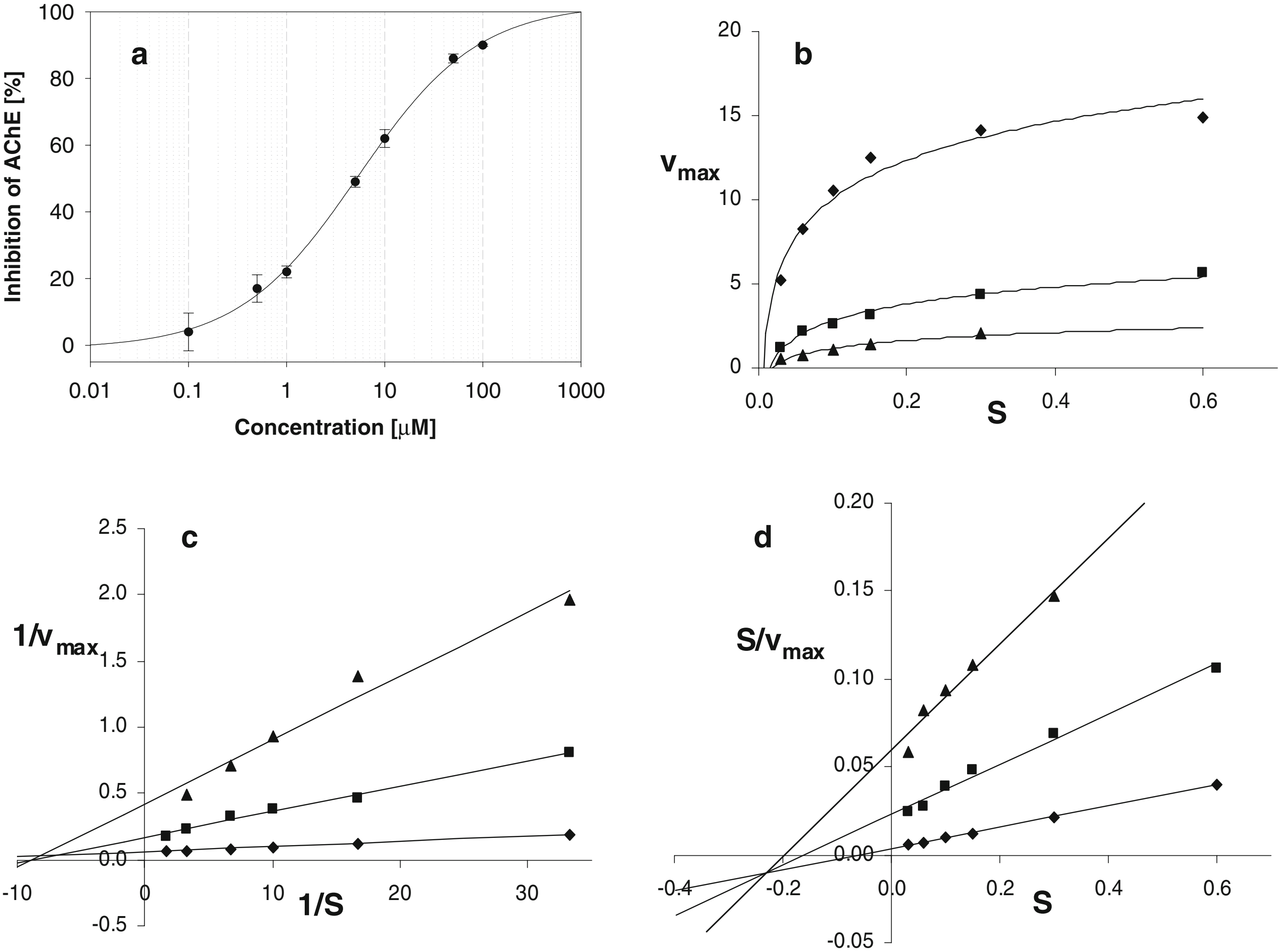

Fig. 3 **a** Concentration-dependent nonlinear regression of the inhibition of acetylcholinesterase (AChE) by nostocarboline iodide (mean of three replicates, R^2=0.99). **b**–**d** In vitro maximal enzyme activity, v_{max} (Δ absorbance/s) of AChE (0.0015 U) with increasing concentrations of substrate S (acetylthiocholine iodide, mM) and with 10 μM (■), 20 μM (▲) and without (♦) nostocarboline iodide. The illustration shows the Michaelis-Menten plot (**b**), the Lineweaver-Burk plot (**c**) and the Hanes plot (**d**). R^2 for the different linear and nonlinear regression lines was > 0.95

Carbolinium ions have been found in the human brain and have been discussed as endogenous causative factors of PD (Matsubara et al. 1993). Kuhn et al. (1996) suggested that β-carbolines such as harmane and norharmane might be neurotoxins causing PD. Gearhart et al. (2002) found β-carboline 2*N*-methyltransferase activity in the mammalian brain. This enzyme converts β-carbolines, like harmane and norharmane, into 2*N*-methylated β-carbolinium cations, which are analogues of the PD-inducing toxin 1-methyl-4-pyridinium cation (MPP^+). A β-carbolinium cation that shares the toxic properties of MPP^+ is the quarternary deschloro-nostocarboline, 2-methylnorharmane, which has been isolated from post-mortem brain tissue (Matsubara et al. 1993).

We have described a similar IC_{50} value for 2-methylnorharmane on BChE (Becher et al. 2005) as that reported on AChE (Ghosal et al. 1972). Subsequently, Schott et al.

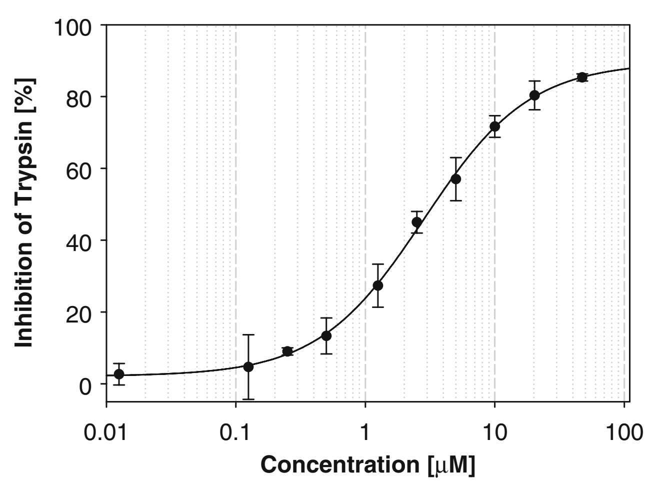

Fig. 4 Concentration-dependent sigmoidal regression of the inhibition of trypsin by nostocarboline iodide (R^2=0.99). Each concentration was tested in three replicates

(2006) tested additional β-carbolines and confirmed our finding of the inhibitory potency for quarternary β-carbolines in the same concentration range as for galanthamine, an approved drug for treatment of AD. Interestingly, tertiary carbolines such as the brunneins are much less active as cholinesterase inhibitors (Teichert et al. 2007).

The cholinergic hypothesis (Bartus et al. 1982) states that a degeneration of cholinergic neurons and the associated loss of cholinergic neurotransmission leads to a decline in cognitive function as a symptom of AD. Some AChE inhibitors, such as galanthamine, donepezil and rivastigmine, are used as pharmaceuticals to alleviate the symptoms of AD by impeding the hydrolysis of ACh. Pharmaceutical control of AChE with inhibitors seems even more reasonable as the enzyme is also involved in the formation of amyloid beta peptide (Inestrosa et al. 1996; Selkoe 1999). Recently, Hostettmann et al. (2006) reviewed natural inhibitors of AChE and emphasised the importance of screening programs to find additional lead compounds for the treatment of AD. Cholinesterase inhibitors are also of significance for the treatment of several other diseases, such as myasthenia gravis (grave muscle weakness). A summary of the use of cholinesterase inhibitors in anaesthesia, intensive care medicine, emergency medicine and pain therapy is given by Kleinschmidt et al. (2005).

Schott and co-workers (2006) suggested tertiary β-carbolines as potential prodrugs that could be bioactivated by methylation for the treatment of AD. Our assay indicated a non-competitive component in the inhibition of AChE by nostocarboline. Bartolini et al. (2003) found that non-competitive AChE inhibitors like donepezil hinder amyloid beta protein aggregation. In view of the comparable inhibitory activity of nostocarboline (and other β-carbolines) to galanthamine (which is characterised as a competitive inhibitor, see Scott and Goa 2000), these compounds should be considered in the design of new drugs for the therapy of AD.

As the reaction centre of AChE is considered to resemble the catalytic triade of chymotrypsin and other serine proteases (Sussman and Silman 1992), we assumed that nostocarboline might also be an inhibitor of proteases. A similar inhibitory activity on AChE and serine proteases is known for phenylmethylsulfonyl fluoride and other sulfonyl fluorides (Fahrney and Gold 1963; Kraut et al. 2000). We found nostocarboline to be a strong inhibitor of mammalian trypsin. The IC_{50} inhibition value was in the range of serine protease inhibitors from cyanobacteria that belong to the chemical group of cyclic peptides (Baumann et al. 2007), depsipeptides and linear peptides with unusual amino acids (Welker and von Döhren 2006). So far all serine protease inhibitors that have been isolated from cyanobacteria are peptides; nostocarboline is the first example of an alkaloid produced by cyanobacteria showing serine protease inhibition. The interference of nostocarboline in protein metabolism may also be of primary pharmaceutical importance because serine proteases are implicated in processing of the amyloid precursor protein (Grau et al. 2005; Park et al. 2006).

The multifunctional character of nostocarboline might be advantageous for *Nostoc* (78–12A) in resisting attacking organisms. An obvious biological function of cyanobacterial secondary compounds is chemical defence or deterrence against grazers (Carmichael 1992; Codd 1995; Wylie and Paul 1988). Several *Nostoc* strains are resistant to predation. This is probably related to the production of large amounts of sheath material and synthesis of toxins. Many secondary metabolites, including the β-carboline norharmane (Volk 2005, 2007; Volk and Furkert 2006), have already been isolated from *Nostoc* species (reviewed by Dembitsky and Řezanka 2005; Volk and Mundt 2007).

We expected potent neurotoxicity of the AChE inhibitor producing *Nostoc* sp. (78–12A), but only moderate toxic effects were found for nostocarboline when tested on the crustacean *Thamnocephalus platyurus.* No clear toxic effects were found when nostocarboline was tested on chironomids sampled from biofilms of the littoral zone of Lake Zürich, nor when fresh biomass of *Nostoc* sp. (78–12A) was fed for 8 days to larvae of *Chironomus riparius* from a laboratory culture (Becher and Jüttner 2006). The reason for these low effects on chironomids might be due to adaptation and resistance of the grazers. The AChE for the enzyme assay was isolated from a vertebrate (electric eel) and thus possibly differed from the insect AChE from *C. riparius* (for differences between AChE of *Torpedo californica* and *Drosophila melanogaster*, see Harel et al. 2000). Resistance mechanisms against insecticides, such as organophosphates and carbamates, are well known for pests and can arise e.g. through enhanced detoxification by cytochrome or structural modification of the target enzyme AChE (Horowitz and Denholm 2001). The induction of cytochrome P_{450}-dependent monooxygenases by sublethal concentrations of pesticides was also shown for *C. riparius* and *C. tentans* (Sturm and Hansen 1999; Miota et al. 2000).

To be relevant in an ecological context, the effects of a compound must not be lethal. We suppose it possible that the physiological response to nostocarboline only affects parts of the grazer, e.g. the mouthparts. Such an effect might be a deterrent to the grazer and thus protective for the cyanobacterium. Further experiments, such as toxicity testing against other aquatic or terrestrial animals, as well as in vitro testing of nostocarboline on AChE of *Chironomus* and additional test organisms may provide further information about the ecological and neurotoxicological significance of the compound.

The trypsin inhibition property of nostocarboline likely is an additional defence mechanism against grazers. Trypsin-like proteases are major components of the digestive proteases in the guts of invertebrates. Any inhibition of this enzyme leads to starvation of the grazers and hence to reduced grazing pressure. The importance of this effect is in line with the occurrence of peptidic trypsin inhibitors in all inedible cyanobacteria.

Furthermore, the protease inhibitory activity might be directed against photosynthetic organisms competing for light. As (serine) proteases are present in chloroplasts and e.g. involved in processing of the polypeptide D1 in the reaction center of photosystem II (Nair and Ramaswamy 2004; Trost et al. 1997; Liao et al. 2000), the protease inhibitory effect might be involved in the described algicidal activity of nostocarboline (Blom et al. 2006).

The presented activities of the cyanobacterial β-carboline nostocarboline and its potential pharmaceutical and ecological qualities reveal *Nostoc* sp. (78–12A) and other cyanobacteria to be promising sources of lead compounds for the development of new drugs and pesticides/insecticides (as reviewed for other natural acetylcholineesterase inhibitors, Houghton et al. 2006) or novel antifouling agents (reviewed in Gademann 2007).

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